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NEUROPROTECTIVE EFFECT CORILAGIN IN SPINAL CORD INJURY RAT MODEL BY INHIBITING NUCLEAR FACTOR-KB, INFLAMMATION AND APOPTOSIS

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Abstract

Background: Neurological functions get altered in a patient suffering from spinal cord injury (SCI). Present study evaluates the neuroprotective effect of corilagin in spinal cord injury rats by inhibiting nuclear factor-kappa B (NF-κB), inflammatory mediators and apoptosis.

Materials and method: Spinal cord injury was produced by mechanical injury to spinal cord. All the rats were treated with corilagin (10 & 15 mg/kg, ip) for the duration of 4 week. Neurological functions were evaluated in all the rats by Basso,Beattie and Brenahan scale (BBB scale). Moreover, at the end of treatment rats were sacrificed and expression of NF-κB, concentration of cytokines like Tumor necrosis factor α (TNFα) & interlukin-6 (IL6) and oxidative stress parameters like Malondialdehyde (MDA) and toal antioxidant capacity (TAC) in spinal tissue of spinal cord injured rats. Immunohistological study was also performed to assess the apoptosis of neuronal cell.

Result: There were significant ($p<0.01$) increase in BBB score and decrease in delayed response to pain in corilagin treated group compared to SCI group. It was also observed that treatment with corilagin significantly ($p<0.01$) decreases the concentration of TNF α, IL 6 & MDA in spinal tissues than SCI group. Moreover, it also decreases the TAC in spinal tissues compared to spinal cord injured rats and also ameliorates the altered expressions of NF-κB. Immunohistochemical result suggested that treatment with corilagin decreases the number of caspase-3 & Bax positive stain cell and increase in Bcl-2 positive stained cell than spinal cord injured rats. Thus, corilagin decreases the apoptosis of neuronal cell in spinal cord injured rats.

Conclusion: This study concludes the neuroprotective effect of corilagin in spinal cord injured rats by decreasing cytokines, oxidative stress & expression of NF-κB in spinal tissues and thereby decreases the apoptosis of neuronal cells.

Keyword: Corilagin, spinal cord, inhibiting nuclear factor-kappa B (NF-κB), apoptosis

Introduction

Spinal cord injury is majorly caused by trauma/accident (Sekhon and Fehlings, 2001). It involves two mechanisms of neuronal cell damage, one is direct injury by physical damage and other is secondary injury through increased inflammatory mediators, free radicals and altered release of neurotransmitters (Popovich and Jones, 2003). Spinal cord injury results in severe disability as it damages the nervous system and thereby alters the neurological functions. All these factors contribute in progression of apoptosis and necrosis process in neuronal cell (Beattie et al., 2002).

Moreover, after mechanical injury the rupture of blood vessel activate the cytokines in the region of spinal cord. Inflammation is one of the major factors responsible for secondary injury to spinal cord. Evidences show the inflammatory mediators (TNFα and IL-6) and oxidative stress induces the process of apoptosis by enhances the activity of NF-κB

signaling in neuronal cells (Kabe et al, 2005; Zou & Crews, 2005). The drug that inhibits NF-κB factor restores the neuronal function in spinal cord injury. However mechanical injury to neuronal fibers in spinal cord injury need not required any therapeutic intervention but secondary injuries are required therapy of drug to prevent the same.

Corilagin is isolated from *Caesalpinia coriaria* herbal and chemically known as ellagitannin (Schmidt & Lademann, 1951). Previously reported studies suggested that corilagin is a potent antioxidant, hepatoprotective, anti inflammatory, analgesic, antihypertensive, antitumor and carbonic anhydrase inhibitor property (Jia et al., 2013; Jin et al., 2013; Kinoshita et al, 2007; Lin et al., 1993). On the basis of its anti-inflammatory, antioxidant and carbonic anhydrase inhibiting activity, present study evaluates the neuroprotective effect of corilagin on SCI in animal model.

Material and Methods

Animal

Healthy female Wistar rats (200-250g) were used in the given investigation procured from Experimental Animal Center of Zhengjiang Province (SYXK 2015-00012). All the rats were acclimatized for a week before the start of study to the laboratory condition. Rats were kept under controlled condition as per the guidelines with pallet feed and water ad libitum. All the protocol of this study was approved by institutional animal ethical committee of The First Affiliated Hospital of Henan University of science and technology (approval ref. no. 10/2015).

Injury to Spinal Cord Induction

Induction of spinal cord injury was achieved as per the previously reported procedure (Gruner, 1992). In which rats were anesthetized by pentobarbitone at a dose of 40 mg/kg of ip injection. Hairs were removed and vertebra was exposed by giving a 20mm of incision at thoracic region. Dorsal cord surface was exposed for laminectomy at T10, in which dissection forceps was used to clear the muscle by penetrating it into paravertebral muscle fascia. Angled clamps were applied specifically on T8 & T12 to stabilize the vertebrae. SCI was produced by dropping a rod of 10 gm weight vertically from a distance of 12.5 mm to the exposed area of vertebral column. After the injury incision was closed by using suture. Rats were placed on a heating pad at a controlled temperature of 37◦C and monitored the same until recovered from anesthesia. In same operated group surgery was performed without induction of injury.

Experimental Design

All the rats were separated into four groups each group carries 10 rats which are as follows. Shame operated (Control), Rats were subjected to spinal cord injury (SCI), corilagin 10 mg/kg, ip treated group (SCI+ corilagin 10 mg/kg), corilagin 15 mg/kg, ip treated group (SCI+ corilagin 15 mg/kg). All the groups were treated with drugs as per given in protocol for the duration of 4 weeks from the day of induction of SCI.

Estimation of neurological function

Neurological function was assessed with open field motor test and it was estimated by using Basso, Beattie and Brenahan scale. BBB scale scoring is depending on different aspects of hind-limb function like stepping ability, weight support and coordination. It is a 21-point scale, in which lowest end i.e. 1 indicates cease of movement and the highest end i.e. 21 indicates the normal function of hind limb. The score was observed on day 1, 7, 14, 21 & 28 of treatment protocol. Estimation of pain was achieved through behavior study by hot water test. Score of it was observed at the end of each week from the day one of injury.

Estimation of electrophysiology

Electrical potential was estimated by inserting electromyography (EMG) recording needle to the hind-limb muscle. In the beginning 10 s electric potential was determined later it was compressed to 1 s to estimate the recruitment index.

Tissue preparation of spinal cord

At the end of protocol all the rats were sacrificed and spinal cord was isolated. Isolated spinal cord was homogenized at 4^{o} C with 7.4 pH phosphate buffer solution. Tissue homogenate was centrifuged at 3000 rpm for the duration of 20 min. Supernatant solution was separated and kept at -80^{o} C until samples used for the estimation of biochemical parameters.

Estimation of cytokines and oxidative stress in spinal cord

Estimation of cytokines such as IL6 and TNFα in spinal cord tissue was achieved by ELISA kit (WKEA MED Supplies, New york, USA). The concentration of protein was determined as given by Lowry et al. Total antioxidant capacity was estimated in spinal cord injured tissues by reacting tissue antioxidant with known quantity of hydrogen peroxide (H_2O_2). Amount of antioxidant present in tissues decreases a specific quantity of H_2O_2 and remaining H_2O_2 reacted with a enzymatic reaction that convert 3, 5, dichloro-2-hydroxy benzsulphonate to a specific color. The quantity of H_2O_2 was estimated by using colorimeter. Moreover, in the given study concentration of malondialdehyde (MDA) in tissue homogenate was estimated by colorimetric kit (Biodiagnostic, Beijing, china) (Lowry et al, 1951; Motterlini et al, 1996).

Estimation of NF-κB in spinal cord

NF-κB protein expression was estimated in spinal cord as a section 3-5 µm of it was take out. Immunostaining was performed by streptavidin–biotin-immunoenzymatic antigen detection method and antibody of NF-κB was also used for detection. All the images were captured and studied through Olympus BX53 microscope associated a digital camera with it (Olympus, Tokyo, Japan). Activity of NF-κB was estimated through counting of cytoplasmic and nuclear positive neuron separately and their percentage compared to total number of neurons.

Estimation of Caspase-3, Bcl-2, and Bax in spinal cord

Spinal cord tissue was embedded in the paraffin and cut it in to sections. Sections of spinal cord deparaffinized and incubated it for the duration of 10min with H_2O_2 in methanol. All the slides were rinsed with PBS for the duration of 5 min and thereafter incubated with goat serum at room temperature for the duration of 10 min. later these samples were kept for incubation with antibodies such as caspase 3, Bax & Bcl2 for overnight at 4°C and then wash it 3 times with PBS for 5min. It was again incubated with secondary antibody for a period of 30min. the TS was stained by using 3,3′-diaminobenzidine tetrahydrochloride with 0.01 % v/v H_2O_2 (pH 7.4) and wash it PBS after desired staining was achieved.

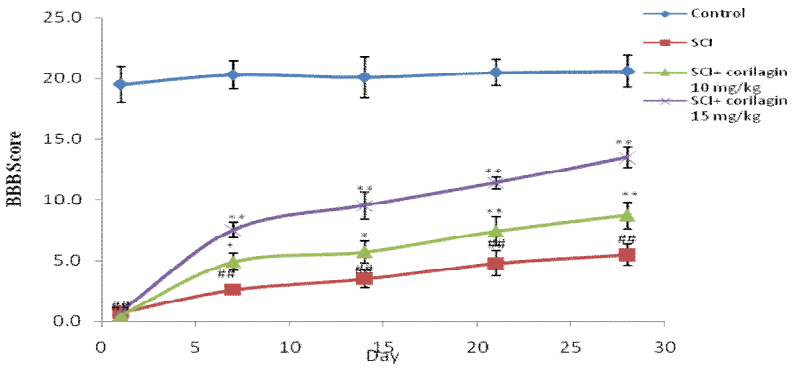
Statistical analysis

All the values of these experiments were articulated as mean ± SD and the data was statistically analyzed by one-way ANOVA and thereafter applied to Dunnett post hoc test. $p<0.05$ was considered significant statistically.

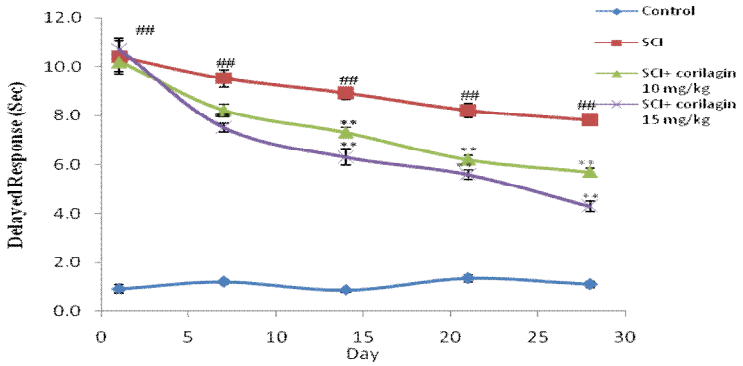
Result

Effect of corilagin on neurological behavior

Spinal cord injury results in the development of disturbance in neurological functions. The effect of corilagin on neurological function in spinal cord injured rat was shown in Fig.1. There were significant ($p<0.01$) decrease in BBB score in SCI group compared to control group. Whereas treatment with corilagin 10 & 15 mg/kg of dose significantly increase ($p<0.05$, $p<0.01$) in the BBB score on 7th, 14th, 21st & 28th day of protocol compared to SCI group as shown in Fig. 1. A. Moreover delayed in response to pain sensation was found to be increases in SCI group compared to control group of rats throughout the tenure of protocol. This increase in the delay in response to pain sensation ameliorated by the treatment with corilagin and this decrease in delay in response to pain sensation was found to be more with the increase in the period of treatment as shown in Fig.1B.



(A)



(B)

Figure 1: Estimation of neuronal function in spinal cord injured rats; (A) Locomotion activity; (B) Hot water test
Data of this study were expressed as mean ± SD; Vs Control, $^{\#\#}p < 0.01$; Vs SCI, $^{**}p < 0.01$

Effect of corilagin on electrophysiological activity

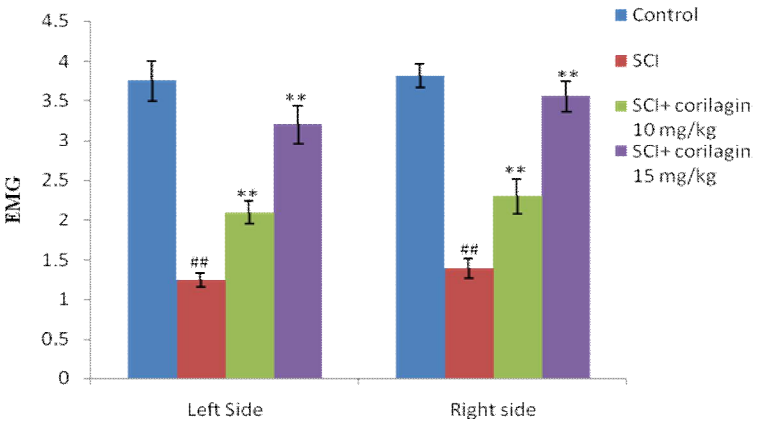


Figure 2: Evaluation of corilagin on electrophysiological factor in spinal cord injured rats
Data of this study were expressed as mean ± SD; Vs Control, $^{\#\#}p < 0.01$; Vs SCI, $^{**}p < 0.01$

Result of this study suggested that recruitment index SCI decreases the same compared to control group. Treatment with corilagin significantly ($p<0.01$) improves the electrophysiological factor for left and right both the hindlimb compared to SCI group of rats. This improvement with corilagin treatment was found to be in a dose dependent manner as shown in Fig. 2.

Effect of corilagin on cytokines and oxidative stress in spinal cord tissues

Concentration of cytokines such as IL-6 & TNF-α and oxidative stress parameter such as MDA & TAC were estimated in spinal cord tissue of spinal cord injured rat as shown in Fig 3. Study suggested that there was significant increase ($p<0.01$) in the concentration of IL-6 & TNF-α in spinal cord tissue of SCI group compared to control group. Whereas, corilagin decreases the concentration of IL-6 & TNF-α in spinal tissue than spinal cord injured rat (Fig. 3 A&B). Moreover, spinal injury provokes the oxidative stress that result in the development of secondary injury to spinal cord. The result of this study suggested that there was significant increase in the MDA and decrease in the TAC level in the spinal tissues of SCI rats in comparison to control group. Treatment with corilagin significantly decreases the concentration of MDA and increases the TAC in spinal tissues than SCI group (Fig 3. C&D)

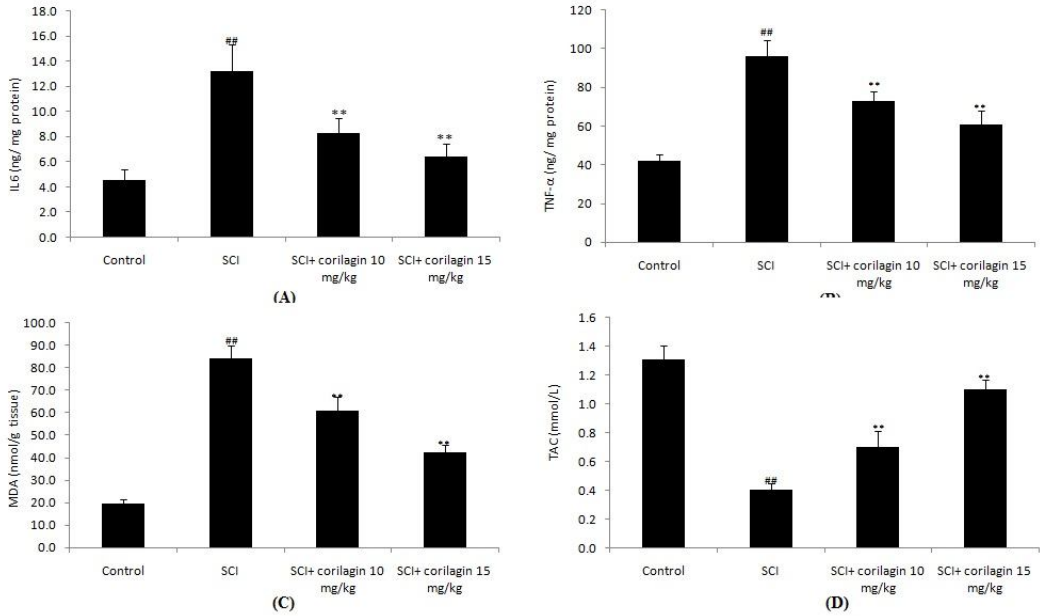
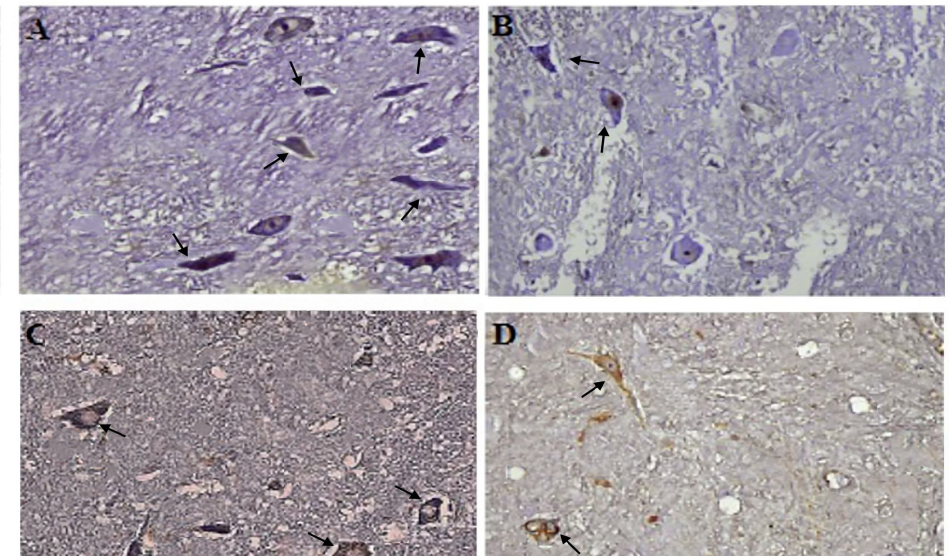


Figure 3: Estimation of cytokines and oxidative stress in spinal cord tissues of spinal cord injured rats; (A) IL-6; (B) **TNF-α; (C) MDA & (D) TAC**

Data of this study were expressed as mean ± SD; Vs Control, $^{\#\#}p < 0.01$; Vs SCI, $^{**}p < 0.01$

Effect of corilagin on NF-κB in spinal cord



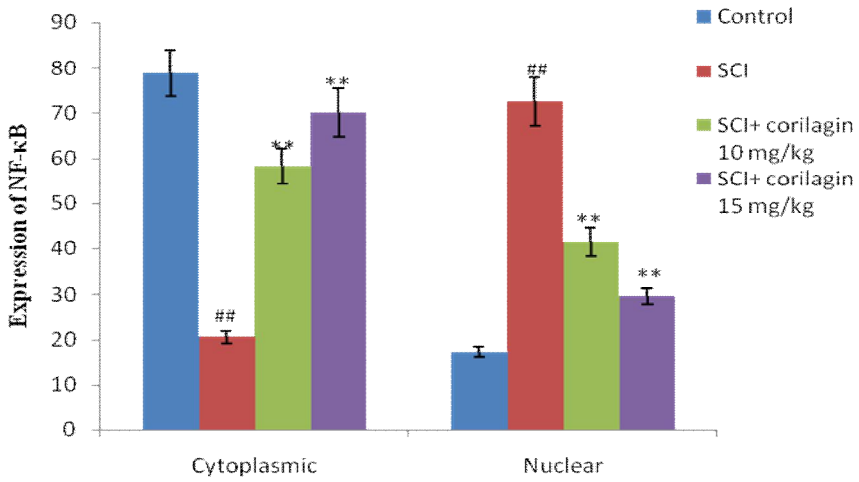


Figure 4: Estimation of NF-κB by immunehistochemical staining in spinal cord of spinal cord injured rat (Magnification 400×); A) Control; B) SCI; C) SCI + corilagin 10 mg/kg; D) SCI + corilagin 15 mg/kg
Data of this study were expressed as mean ± SD; Vs Control, $^{\#\#}p < 0.01$; Vs SCI, $^{**}p < 0.01$

Immuno-histochemical expression of NF-κB protein was more in cytoplasmic and less in nuclear in normal rat. Spinal cord injury results in significant increase in expression of NF-κB protein in nucleus and decrease in cytoplasm. In corilagin treated group, expression of NF-κB protein was found more in cytoplasma and decrease in nuclei. This result in amelioration of altered expression of NF-κB protein in neuronal of spinal cord injured rat by corilagin in a dose dependent manner as shown in Fig. 4.

Effect of corilagin on apoptosis of neuronal cells

Effect of corilagin on apoptosis neuronal cell was shown in Fig. 5. Here it was observed that treatment with corilagin significantly decreases in the TUNEL positive cell compared to SCI group. Immuno-histochemical staining was done to assess the effect of corilagin on caspase 3, Bax and Bcl2 in spinal cord tissue of SCI group of rats. There were significant decreased in caspase 3 & Bax positive cell in corilagin treated group compared SCI group of rats. In contract the Bcl2 positive cell quantity was found to be increases in corilagin treated group than spinal cord injured rats.

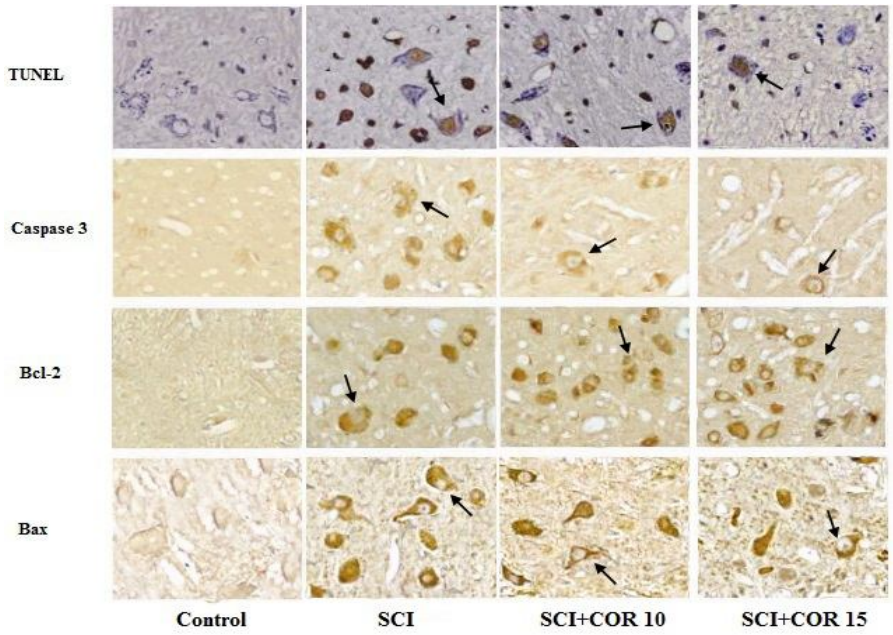


Figure 5: Estimation of corilagin on immunohistochemical staining of TUNEL, caspase-3, Bcl2 & Bax of spinal cord tissue in spinal cord injured rat

Discussion

Present investigation evaluates the protective effect of corilagin on neuronal cell in spinal cord injured rats. It reduces secondary spinal cord injury by decreasing cytokines, oxidative stress and expression of NF-κB protein in spinal cord tissue and thereby decreasing apoptosis of neuronal cell.

Spinal cord injury is a very complex neuropathological condition which involves various molecular processes (Dumont et al., 2001). Primary spinal cord injury by trauma develops some events like increased calcium overload & oxidative stress & concentration of cytokines (Aksenova et al., 2002). These altered parameters like increased oxidative stress result in development of secondary injury by decreasing membrane enzyme activity and increased activity of cytokines such as TNFα & IL (Martin and Liu, 2002). It was observed that in this study that corilagin significantly ($p<0.01$) decreases the oxidative stress by increasing the total antioxidant capacity and decreasing MDA in the spinal tissue homogenate of spinal cord injured rat compared to SCI group. Corilagin treatment also decreases the concentration of cytokines in the spinal tissue than spinal cord injured rats. Drug with strong antioxidant activity is effectively used in the management of spinal cord injury and known to have neuroprotective effect (Kaka et al., 2016).

Recent study suggested that apoptosis plays a role in the development of secondary spinal cord injury (Emery *et al*., 1998). Oxidative stress and inflammatory mediators triggers the activity of caspase cascade and result in increased activity of caspase 3, Bax & decrease in Bcl-2 in injured spinal cord tissues. Alteration of these molecular factors promotes the apoptosis which result in neuronal cell death promotion and alteration of neuronal function (Hengartner, 2000). Moreover, in spinal cord injured tissue expressions of NF-κB protein get altered and there was a close relation between oxidative stress, cytokines, NF-κB and apoptosis in secondary injury induced neuronal damage. Literature suggested that by decreasing the expressions of NF-κB protein inhibit the apoptosis induced in spinal cord injured rats (Engelmann et al, 2014). Immunohistochemical study of spinal tissue suggested that there was significant decrease in caspase 3 & Bax positive stained cell in corilagin treated rats compared to spinal cord injured rats. In contrast, the number of Bcl2 positive cells was increased with its treatment than spinal cord injured rats. Moreover, corilagin significantly ameliorates the expressions of NF-κB protein that was altered by spinal cord injury.

Present study concludes the neuroprotective effect of corilagin in spinal cord injured rat model. It inhibits the apoptosis of neuronal cell by decreasing oxidative stress, inflammatory mediators & NF-κB in spinal cord injured tissue.

Conflicts of Interest: The authors declare that this research presents no conflicts of interest.

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